HypoxiTRAK™ TECHNICAL DATA SHEET

PRODUCT: HypoxiTRAK™ **PRESENTATION:** aqueous solution.
PRODUCT NUMBERS: HT10500 **STORAGE**: store at 2-8 ºC. Do not freeze.

DESCRIPTION:
HypoxiTRAK™ is a novel, far-red fluorescing dye that reveals hypoxic experience of individual cells yet is non-toxic to normoxic cells. HypoxiTRAK™ suits flow cytometry and imaging protocols and compatible with common vis. range fluors e.g. FITC, R-PE. HypoxiTRAK™ enables entirely new assay approaches to functional hypoxia.

APPLICATIONS:
Reporting hypoxia experience in cell cultures, spheroids and tissues:
- Flow Cytometry – dynamic live cell hypoxia study
- Fluorescence Microscopy – dynamic live cell hypoxia study; immuno-fluorescence

BACKGROUND INFORMATION:
Hypoxia's role in cancer and stem cells is an expanding research area. Low oxygen stress can alter biological behaviour and pharmaco-dynamic responses via hypoxia-induced activation of cell-protective and proliferation mechanisms.

HypoxiTRAK™ is a novel molecular probe for use in flow cytometry and imaging to report (i) degree of hypoxic experience of cells and (ii) functionally hypoxic fraction of cells. HypoxiTRAK™ is designed to activate by bio-reduction at biologically relevant levels of hypoxia, since *in vivo* tumour cells can occupy hypoxic niches with lower median oxygen levels (~1 % oxygen; pO_2 7.5 mmHg) compared to normal tissues (~5.5% oxygen; pO_2 42 mmHg) while tumour cores may maintain a less than 0.1% oxygen (pO_2 1 mm Hg) environment. Hypoxia sensing range for HypoxiTRAK™ is relevant to biomarker and hypoxia-targeting drug development.

The bioactive metabolite retains the parent fluorophore and accumulates in cells, proportional to the hypoxia experienced, to provide a persistent far-red fluorescent signature and induces cell arrest, thereby marking and 'freezing' the hypoxic cells, enabling a direct read-out of a hypoxic cell fraction.

HypoxiTRAK™ allows assessment of degree of hypoxia by simple assays for growth arrest or apoptosis. HypoxiTRAK™ bio-activation, resulting in prolonged cell cycle arrest, can be detected by increased side scatter by flow cytometry. As no fixation is required, HypoxiTRAK™ provides a convenient negative selection for cells not experiencing relevant hypoxia within a heterogeneous population, including 3D culture. HypoxiTRAK™ shares spectral features with the DNA dye DRAQ5™, that labels all nucleated cells, a convenient standard to mark all cells within a sample in parallel with analysis for hypoxic fraction.

HypoxiTRAK™ is typically present during the period of hypoxia under investigation. The physicochemical properties of HypoxiTRAK™ facilitate permeation throughout populations of cells in 2D and 3-D culture (2) and tissues thereby enhancing its entry into hypoxic niches and reducing retention upon cell isolation, providing a good signal:noise. HypoxiTRAK™ is ideal for studies over several days. HypoxiTRAK™ read-out is direct, obviating cell processing. HypoxiTRAK™ shows low perturbation in the absence of bio-activation while the unique far-red fluorescence signature offers compatibility with other fluorescent end-point assays for hypoxia.

Oxygen levels vary in extent and duration; HypoxiTRAK™ provides a retrospective / historical reporting of the integrated hypoxic experience of a cell – it is a reporter of the hypoxia experienced by each cell within an *in vitro* cell population.

BEFORE STARTING:
Read the MSDS. Wear protective clothing, safety goggles and laboratory gloves.
Check the concentration of HypoxiTRAK™ stated on the vial label.

MATERIALS OFTEN REQUIRED BUT NOT SUPPLIED:
PBS (azide-free), culture medium (CM), CM without phenol-red ("Imaging CM"), Paraformaldehyde, Antibodies.

SPECTRAL CHARACTERISTICS:
Exλ_{max} 600/646 nm Emλ_{max} 697 nm

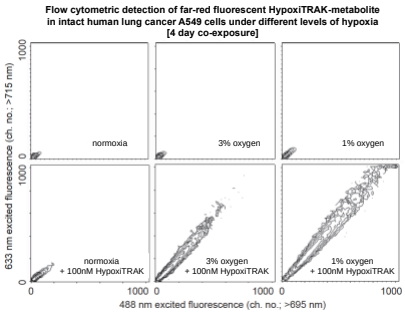

Fig. 1 Flow cytometry of HypoxiTRAK™-metabolite, blue & red excitation; intact A549 cells under hypoxia (3%, 1%), with & without HypoxiTRAK™ [4 day co-exposure]

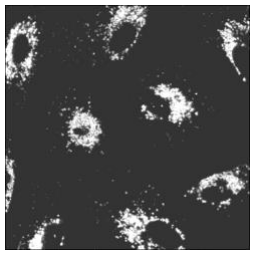

Fig. 2 HypoxiTRAK™-metabolite accumulates in cytoplasm of A549 cells (ex. 633 nm; em. 680/20 nm) exposed to 100 nM HypoxiTRAK™ for 4 days 1% O_2

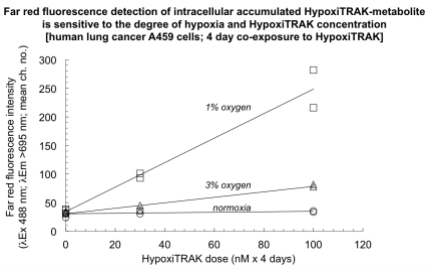

Fig.3 Intracellular accumulation of HypoxiTRAK™-metabolite is sensitive to degree of hypoxia and [HypoxiTRAK] (A549 cells, conditions as fig. 2)

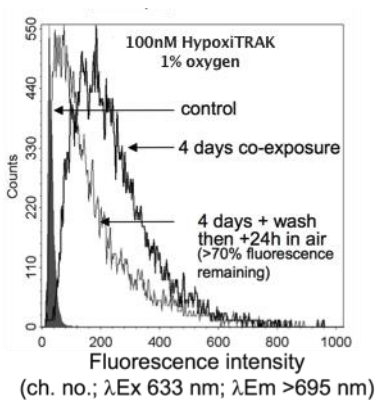

Fig. 4 HypoxiTRAK™-metabolite persistence: intact A549 cells retain >70% after washing and 24h incubation in HypoxiTRAK™-free medium (ex. 633nm; em. >695nm)

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****NOTE:** retained HypoxiTRAK™-metabolite inhibits cell division - marking and preserving cells that have experienced hypoxic conditions.

DETECTING HypoxiTRAK™ SIGNALS: (see Fig. 1)
Flow cytometry: HypoxiTRAK™-metabolite can be excited by 488 or 635 nm wavelength light. Detect in the 670LP channel or similar bandpass filters centred on 700 nm.
Microscopy / HCS Imaging Platform: HypoxiTRAK™ is optimally excited using yellow / red wavelengths. Detect with far-red filters >660 nm e.g. Cy5 filter on HCS imager, or Chroma Part No. 49019 (Ex. 620/20nm, Em. 665LP, HQ 700/75)

EXAMPLE PROTOCOL
To report hypoxia, HypoxiTRAK™ is added to CM at the appropriate time in the protocol. It does not need to be replaced for analysis of the cells.

NOTE: *for image-based analysis - phenol red indicator contributes to background fluorescence and should be avoided or any CM containing it replaced by the equivalent Imaging CM prior to imaging.*

1. Prepare cell cultures with treatments, if any, to be subjected to hypoxia (t = 0): monolayer in early growth phase or suspension culture at approximately 2 x 10^5 cells/ml. If required harvest a parallel culture at the start of the experiment to established culture density.
2. Add HypoxiTRAK™ directly to cultures: initially use a final concentration range of 10, 30, 50, 100 nM HypoxiTRAK™ to test for dynamic range with respect to the prevailing culture density and extent of hypoxia.
   NOTE: If procedures require it, make up the diluted (i.e. working conc$^{n.}$) HypoxiTRAK™ required for up to one day's lab work e.g. total volume required to set up a set of wells.
3. Incubate cultures under the selected hypoxic conditions for chosen periods (2-7 days typically) during which biologically relevant changes to cell behaviour are under study.
4. Analysis:
   For adherent cultures – wells can be time-lapse imaged over several days, or cells harvested by an appropriate method, washed to remove debris and re-suspended in cold medium prior to conventional flow cytometry.
   For suspension cultures - samples can be used directly without any processing and analysed by flow cytometry.
   For 3D microtissues (spheroids, organoids, MCTS, etc.) – wells can be time-lapse imaged over several days from cell aggregation or from another appropriate stage in cell culture progress.

   Samples (e.g. suspension cells, adherent cells, cryo-preserved sections) requiring further staining for immunofluorescence should be fixed with 4% formaldehyde for 5 minutes. Care should be taken to limit PBS washes to a minimum to avoid wash-out of HypoxiTRAK™-metabolite, prior to immune-staining.

   Cell enumeration will reveal reciprocity between cell proliferation capacity, viability and HypoxiTRAK™ bioactivation.

WHAT YOU SHOULD EXPECT TO SEE
In cells that have experienced functional hypoxia, HypoxiTRAK™ accumulates in the cytoplasm (see fig. 2), detected by fluorescence microscopy or by flow cytometry (figs. 1, 3, and 4). 3D microtissues can be imaged at 10x magnification to show patterning of hypoxic experience throughout the object. Flow cytometric data for each time point should be displayed as Cumulative Distribution Function (CDF) compared to the control(s). As a far-red fluorescing dye HypoxiTRAK™ signals are spectrally separated from the majority of visible-range chromophores.

REFERENCES
1. O'Connor, Liam J., et al. "CYP450 enzymes effect oxygen-dependent reduction of azide-based fluorogenic dyes." *ACS central science* 3.1 (2017): 20-30.
2. Close, David A., and Paul A. Johnston. "Detection and impact of hypoxic regions in multicellular tumor spheroid cultures formed by head and neck squamous cell carcinoma cells lines." *SLAS Discovery* (2021). DOI:10.1016/j.slasd.2021.10.008

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HypoxiTRAK™ is a trademark of BioStatus Limited.

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